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<b>(21) International Application Number:</b> PCT/US98/17637 <b>(22) International Filing Date:</b> 21 August 1998 (21.08.98)  <b>(30) Priority Data:</b> 08/916,166                      21 August 1997 (21.08.97)                      US  <b>(71) Applicant:</b> POWDERJECT VACCINES, INC. [US/US]; 585 Science Drive, Madison, WI 53711 (US).  <b>(72) Inventor:</b> McCABE, Dennis, E.; 8777 Airport Road, Middleton, WI 53562 (US).  <b>(74) Agent:</b> SEAY, Nicholas, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MUCOSAL IMMUNIZATION USING PARTICLE-MEDIATED DELIVERY TECHNIQUES  <b>(57) Abstract</b>  A method for eliciting an immune response against a virus or other pathogens in a mammalian subject is provided. The method includes the steps of providing a particle coated with DNA encoding an antigen derived from a virus, and then administering the particle to mucosal tissue of the mammal using particle-mediated delivery techniques, whereby the particle is delivered into a recipient cell in said tissue. The technique is capable of inducing an effective mucosal immune response in mammals.		



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MUCOSAL IMMUNIZATION USING  
PARTICLE-MEDIATED DELIVERY TECHNIQUES

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR  
DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Technical Field

15 The present invention relates generally to methods of immunization. More particularly, the invention pertains to the delivery of nucleic acid molecules or peptide antigens into mucosal tissue using particle-mediated delivery techniques.

20 Conventional vaccination strategies generally involve administration of either "live" or "dead" vaccines. Ertl et al. (1996) *J. Immunol.* 156:3579-3582. The so-called live vaccines include attenuated microbes and recombinant molecules based on a living vector. The dead vaccines include those based on killed whole pathogens, and subunit vaccines, e.g., soluble pathogen subunits or protein subunits. Live vaccines are generally successful in providing an effective immune response in immunized subjects; however, such vaccines can be dangerous in immunocompromised or pregnant subjects, can revert to pathogenic organisms, or can be contaminated with other pathogens. Hassett et al. (1996) *Trends in Microbiol.* 8:307-312. Dead vaccines avoid the safety problems associated with live vaccines; however such vaccines often fail to provide an appropriate and/or effective immune response in immunized subjects.

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5           More recently, direct injection of DNA and mRNA into  
mammalian tissue for the purpose of eliciting an immune  
response has been described. See, e.g., U.S. Patent No.  
5,589,466. The method, termed "naked DNA immunization,"  
10       has been reported to elicit both humoral and cell-mediated  
immune responses following DNA delivery to muscle. For  
example, sera from mice immunized with a human  
immunodeficiency virus type 1 (HIV-1) DNA construct  
encoding the envelope glycoprotein, gp160, were reported to  
15       react with recombinant gp160 in immunoassays and  
lymphocytes from the injected mice were shown to  
proliferate in response to recombinant gp120 (Wang et al.  
(1993) *Proc. Natl. Acad. Sci. USA* 90:4156-4160), and mice  
immunized with a plasmid containing a genomic copy of the  
human growth hormone (hGH) gene demonstrated a humoral  
20       immune response (Tang et al. (1992) *Nature* 356:152-154).  
Likewise, intramuscular injection of DNA encoding  
influenza nucleoprotein has been shown to elicit a CD8+ CTL  
response that can protect mice against subsequent lethal  
challenge with virus. Ulmer et al. (1993) *Science*  
25       259:1745-1749. Immunohistochemical studies of the  
injection site revealed that the DNA was taken up by  
myeloblasts, and cytoplasmic production of viral protein  
could be demonstrated for at least six months. Therefore,  
these immunization techniques can be used to provide for  
30       the *in vivo* synthesis of antigenic proteins in a manner  
that is consistent with natural infection. Such endogenous  
production allows for processing of the antigens along the  
classical MHC class I pathway and presentation to CD8+ T  
lymphocytes, as well as uptake and presentation of soluble  
35       proteins by MHC class II molecules to CD4+ T lymphocytes.  
These features induce both cellular and humoral immune  
responses, allowing nucleic acid immunization to provide  
the immunogenic advantages of live vaccines without the  
concomitant safety concerns. However, the technique of  
40       injection of naked DNA into muscle is relatively  
inefficient and requires much more DNA than other DNA

5       vaccination approaches.

          A number of delivery techniques can be used to deliver  
nucleic acids for immunizations, including particle-  
mediated (gene gun) techniques which accelerate nucleic  
acid-coated microparticles directly into the interior of  
10       cells in the target tissue. Gene gun-based nucleic acid  
immunization has been shown to elicit both humoral and  
cytotoxic T lymphocyte immune responses following epidermal  
delivery of nanogram quantities of DNA. Pertmer et al.  
(1995) *Vaccine* 13:1427-1430. Particle-mediated delivery  
15       techniques have been compared to other types of nucleic  
acid inoculation, and found markedly superior. Fynan et  
al. (1995) *Int. J. Immunopharmacology* 17:79-83, Fynan et  
al. (1993) *Proc. Natl. Acad. Sci. USA* 90:11478-11482, and  
Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523.  
20       Such studies have investigated particle-mediated delivery  
of nucleic acid-based vaccines to both superficial skin and  
muscle tissue. One possible reason for the markedly better  
results achieved with the gene gun is that the DNA is  
delivered intracellularly as opposed to the extracellular  
25       delivery by intramuscular injection.

          The immunity mechanisms provided by humoral and  
mucosal immunity systems differ significantly. Mucosal  
immunity provides an important first line of defense in  
protection against pathogens which enter through mucosal  
30       tissues. The mucosal surfaces of the gastrointestinal,  
respiratory and genitourinary tracts are continuously  
exposed to foreign antigen, including potentially  
infectious bacterial, viral and sometimes parasitic  
organisms. Mucosal immune responses may protect against  
35       such challenges, and have distinct and specialized  
characteristics. Holmgren et al. (1994) *Am. J. Trop. Med.*  
*Hyg.* 50:42-54. Mucosal immunity includes both a humoral  
(antibody) response and a cytotoxic T lymphocyte (CTL)  
response, similar to non-mucosal immunity except localized  
40       to mucosal tissue.

          The current dogma holds as follows. 1. The principal

5 immunoglobulin produced by the mucosal immune system is  
secretory IgA, which is the most abundant immunoglobulin  
class in humans. 2. Specialized antigen uptake cells in  
the Peyer's Patches of intestinal tract or nasopharyngeal  
10 lymphoid tissues, termed microfold or M cells, transport  
antigen to the underlying mucosal associated lymphoid  
tissues (MALT). 3. In other areas of the mucosal  
epithelium, such as the pseudo-stratified airway  
epithelium, dendritic cells serve as antigen-presenting  
15 cells and migrate to local lymph nodes or MALT. Antigen  
processing and presentation occurs in the MALT, resulting  
in activation of antigen-specific IgA B cells. The  
subsequent trafficking and recirculation of the activated  
IgA-B cells to other components of the mucosal immune  
20 system, e.g., the respiratory, intestinal and genital  
tracts, provides for disseminated local mucosal IgA  
responses throughout the "Common Mucosal System." Thus,  
the mucosal immune system is uniquely suited to respond to  
the types of antigenic challenge encountered by mucosal  
25 surfaces, and may provide the most effective type of immune  
response against pathogens that initially infect or enter  
the body through mucosal surfaces. It is difficult to  
achieve effective mucosal immune response using most prior  
art techniques.

#### BRIEF SUMMARY OF THE INVENTION

30 The present invention provides an effective method for  
eliciting an immune response in a mammalian subject using  
mucosal immunization and particle-mediated delivery  
techniques.

Accordingly, in one embodiment, the invention is drawn  
35 to a method for eliciting a mucosal immune response or a  
**systemic immune response** against a virus in a mammalian  
subject. The method includes the steps of (a) providing a  
particle coated with a nucleotide sequence encoding an  
antigen derived from the virus, wherein the nucleotide  
40 sequence is operably linked to control sequences that



5       direct the expression thereof in a suitable recipient cell;  
and (b) administering the particle to mucosal tissue of the  
mammal using particle-mediated delivery techniques, whereby  
the particle is delivered into a recipient cell in said  
tissue, and the nucleotide sequence expressed at sufficient  
10       levels to elicit a mucosal immune response against said  
antigen.

In another embodiment, a method includes the steps of  
(a) providing a particle coated with an antigen derived  
from a virus; and (b) administering the particle to mucosal  
15       tissue of the mammal using particle-mediated delivery  
techniques, whereby the particle is delivered into a  
recipient cell in said tissue.

These and other embodiments of the present invention  
will readily occur to those of ordinary skill in the art in  
20       view of the disclosure herein.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a schematic representation of the  
hemagglutinin (HA) expression vector pWRG1638. This  
plasmid vector was constructed from pWRG7054, a mammalian  
25       expression vector based on a pUC19 backbone, and thus  
contains the cytomegalovirus (CMV) immediate early  
transcriptional enhancer, promoter and intron A regulatory  
elements, and the polyA signal of bovine growth hormone,  
operably linked to the full length cDNA encoding the HA  
30       gene from swine influenza virus A/Swine/Indiana/1726/88  
(H1N1).

Figure 2 depicts the geometric mean titers of nasal  
viral shedding profiles in porcine subjects after challenge  
with the swine influenza virus A/Swine/Indiana/1726/88  
35       (Sw/IN) as described in Example 1. The animals were  
vaccinated using nucleic acid immunization by a prime and  
booster administration with: a control plasmid DNA (open  
squares); the pWRG1638 construct to the epidermis (open  
diamond); the pWRG1638 construct to mucosal tissue (open  
40       triangles); or the pFluNP construct to epidermis (open

5 circles). Control animals were vaccinated using parenteral injection by a prime and booster administration with a commercial inactivated whole virus vaccine (crossed squares). All animals were challenged two weeks after the booster immunization.

10 DETAILED DESCRIPTION OF THE INVENTION

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular antigens or to antigen-coding nucleotide sequences. It is also understood that different  
15 applications of the disclosed methods may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

20 All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an", and  
25 "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more such agents, reference to "a particle" includes reference to mixtures of two or more particles, reference to "a recipient cell"  
30 includes two or more such cells, and the like.

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the  
35 invention pertains. The following terms are intended to be defined as indicated below.

An "antigen" refers to any agent, generally a macromolecule, which can elicit an immunological response in an individual. The immunological response may be

5 mediated by B- and/or T-lymphocytic cells. The term may be  
used to refer to an individual macromolecule or to a  
homogeneous or heterogeneous population of antigenic  
macromolecules. As used herein, "antigen" is generally  
10 used to refer to a protein molecule or portion thereof  
which contains one or more epitopes.

A "B cell epitope" generally refers to the site on an  
antigen to which a specific antibody molecule binds. The  
identification of epitopes which are able to elicit an  
antibody response is readily accomplished using techniques  
15 well known in the art. See, e.g., Geysen et al. *Proc.*  
*Natl. Acad. Sci. USA* (1984) 81:3998-4002 (general method of  
rapidly synthesizing peptides to determine the location of  
immunogenic epitopes in a given antigen); U.S. Patent No.  
4,708,871 (procedures for identifying and chemically  
20 synthesizing epitopes of antigens); and Geysen et al.,  
*Molecular Immunology* (1986) 23:709-715 (technique for  
identifying peptides with high affinity for a given  
antibody).

"T cell epitopes" are generally those features of a  
25 peptide structure capable of inducing a T cell response.  
In this regard, it is accepted in the art that T cell  
epitopes comprise linear peptide determinants that assume  
extended conformations within the peptide-binding cleft of  
MHC molecules, (Unanue et al. (1987) *Science* 236:551-557).  
30 As used herein, a T cell epitope is generally a peptide  
having about 3-5, preferably 5-10 or more amino acid  
residues.

"Gene delivery" refers to methods or systems for  
reliably delivering foreign DNA into host cells. Such  
35 methods can result in the expression of the foreign DNA in  
the host cells.

A "nucleotide sequence" or a "nucleic acid molecule"  
refers to single or double stranded DNA and RNA sequences.  
The term captures molecules that include any of the known  
40 base analogues of DNA and RNA.

A "coding sequence" or a sequence which "encodes" a

5 particular polypeptide antigen, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences.

10 The term DNA "control sequences" refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively  
15 provide for the transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected gene is capable of being transcribed and translated in an appropriate recipient cell. The control sequences for  
20 eukaryotes and prokaryotes can differ significantly, and for the present invention eukaryotic, and preferably, mammalian or mammalian virus control sequences are most suitable.

"Operably linked" refers to an arrangement of elements  
25 wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding  
30 sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding  
35 sequence.

#### B. General Methods

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may,  
40 of course, vary. It is also to be understood that the

5 terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

10 In accordance with the present invention, a method is defined for achieving mucosal immunity and/or systemic immunity against an antigen for a pathogen which normally enters the subject body through mucosal tissue. The mucosal immunity, which is contrasted to the humoral immunity obtained through prior DNA vaccination protocols, is achieved by the intracellular delivery of the DNA directly to target mucosal tissues. It has been found that delivery of DNA encoding a pathogenic antigen into the mucosal tissue of a mammal will result in a mucosal immunity expressed by mucosal tissues of the patient including those quite distant from the tissues into which the DNA vaccine is delivered. Prior work has revealed some irregularity in the production of distal mucosal immune response, but the method described here has been found to produce a response in distal mucosal tissues. This enables the DNA vaccine to be delivered to the patient at the site most convenient for delivery of the DNA vaccine, whether or not that site where mucosal tissue is the preferred route of entry of the particular pathogen against which the patient is to be immunized. A systemic immune response as used herein may include a cell-mediated immune response characterized by peripheral blood CTL or a humoral immunity characterized by increased levels of circulating antibodies, such as IgG, in post immunization blood sera.

35 In particular, it has been found that the tongue and the buccal tissue of the interior of the mouth, or cheek, are the most convenient tissues into which to direct a particle-mediated DNA vaccine delivery protocol. The tissues are readily accessible through relatively non-

5       invasive procedures. It has also been found that both  
tongue and buccal tissue are capable of engendering a  
sufficient immune response to introduce mucosal immunity by  
antigen encoding DNA delivered to these tissues. It has  
10       been found that the delivery of antigen encoding DNA to the  
cheek or buccal tissues results in a systemic mucosal  
immune response shared by ?immune? mucosal tissues  
throughout the body.

15       The present invention provides a method for eliciting,  
in a mammalian subject, an immune response against  
mucosally transmitted pathogens using nucleic acid  
immunization and particle-mediated delivery techniques.  
The method can thus be used in a variety of mammalian  
subjects to provide a suitable immune response against  
infection by a pathogen which would normally enter the  
20       subject through a mucosal tissue. Mucosal tissues are the  
preferred entry site into the body for a wide variety of  
pathogens. Pathogens which enter the body through mucosal  
tissues include Human Pappiloma Viruses (HPV), HIV,  
25       HSV2/HSV1, influenza virus (types A, B, and C), Polio  
virus, RSV virus, Rhinoviruses, Rotaviruses, Hepatitis A  
virus, Norwalk Virus Group, Enteroviruses, Astroviruses,  
Measles virus, Para Influenza virus, Mumps virus,  
Varicella-Zoster virus, Cytomegalovirus, Epstein-Barr  
virus, Adenoviruses, Rubella virus, Human T-cell Lymphoma  
30       type I virus (HTLV-I), Hepatitis B virus (HBV), Hepatitis C  
virus (HCV), Hepatitis D virus, Pox virus, Marbug and  
Ebola; bacteria including *M. tuberculosis*, *Chlamydia*, *N.*  
*Gonorrhea*, *Shigella*, *Salmonella*, *Vibrio Cholera*, *Treponema*  
*pallidua*, *Pseudomonas*, *Bordetella pertussis*, *Brucella*,  
35       *Franciscella tulorensis*, *Helicobacter pylori*, *Leptospria*  
*interrogans*, *Legionella pneumophila*, *Yersinia pestis*,  
*Streptococcus* (types A and B), *Pneumococcus*, *Meningococcus*,  
*Hemophilus influenza* (type b), *Toxoplasma gondii*,  
*Compylobacteriosis*, *Moraxella catarrhalis*, *Legionella*  
40       *pneumophila*, *Pseudomonas aeruginosa*, *Donovanosis*, and  
*Actinomyces*; fungal pathogens including Candidiasis and

- 5       Aspergillosis; parasitic pathogens including Taenia,  
Flukes, Roundworms, Amebiasis, Giardiasis, Cryptosporidium,  
Schistosoma, Pneumocystis carinii, Trichomoniasis and  
Trichinosis. The present invention can be used to provide  
a suitable immune response against numerous veterinary  
10       diseases, such as Foot and Mouth diseases, Coronavirus,  
*Pasteurella multocida*, *Helicobacter*, *Strongylus vulgaris*,  
*Actinobacillus pleuropneumonia*, Bovine viral virus diarrhea  
(BVDV), *Klebsiella pneumoniae*, *E. coli*, *Bordetella*  
*pertussis*, *Bordetella parapertussis* and *brochiseptica*.
- 15       The invention is broadly applicable for providing an  
immune response against any pathogen which would normally  
enter through mucosal tissue. In the examples below, there  
is reference to influenza virus and immunodeficiency virus  
DNA. Both of these are intended only as examples of  
20       viruses which enter the body through mucosal tissues. It  
is here thought that a suitable mucosal immune response can  
be created following delivery of DNA encoding antigens from  
these viruses to mucosal tissues. By suitable immune  
response, it is meant that the methods of the invention can  
25       bring about in an immunized subject an immune response  
characterized by the stimulation and clonal expansion of B  
and/or T lymphocytes specific for a virus antigen, wherein  
the immune response can protect the subject against  
subsequent infection with homologous or heterologous viral  
30       strains, reduce viral burden and/or shedding during an  
infection, bring about resolution of infection in a shorter  
amount of time relative to a non-immunized subject, or  
prevent or reduce clinical manifestation of disease  
symptoms.
- 35       Generally, nucleic acid molecules used in the subject  
methods contain coding regions with suitable control  
sequences and, optionally, ancillary therapeutic nucleotide  
sequences. The nucleic acid molecules are prepared in the  
form of vectors which include the necessary elements to  
40       direct transcription and translation in a recipient cell.  
The nucleic acids may be the entire genome of the virus

5 less only sequences necessary for viral pathogenicity.

In order to augment an immune response in an immunized subject, the antigen-encoding nucleic acid molecules can be administered in conjunction with ancillary substances, such as pharmacological agents, adjuvants, cytokines, or in  
10 conjunction with delivery of vectors encoding cytokines.

More particularly, ancillary therapeutic nucleic acid sequences coding for peptides known to stimulate, modify, or modulate a host's immune response, can be coadministered with the above-described antigens. Thus, genes encoding  
15 one or more of the various cytokines (or functional fragments thereof), such as the interleukins, interferons, and colony stimulating factors, will find use with the instant invention. The gene sequences for a number of these substances are known. In one embodiment of the  
20 invention, mucosal nucleic acid immunization is coupled with codelivery of one or more of the following immunological response modifiers: IL-2; IL-4; IL-6; IL-10; IL-12; and IFN- $\gamma$ .

Modes of carrying out the invention are described more  
25 fully below.

#### Isolation of Genes and Construction of Vectors

Nucleotide sequences selected for use in the present invention can be derived from known sources, for example, by isolating the same from infected cells or viral  
30 particles containing a desired gene or nucleotide sequence using standard techniques. The nucleotide sequences for many, if not most, pathogen antigens have been identified to assist in vaccine and therapy design. It is now possible to construct DNA molecules of significant length  
35 once DNA sequence information is available.

Once coding sequences for desired antigens have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an  
40 appropriate cloning vector is a matter of choice.



5        Ligations to other sequences are performed using standard procedures, known in the art.

      Selected nucleotide sequences can be placed under the control of regulatory sequences such as a promoter or ribosome binding site (collectively referred to herein as  
10        "control" elements), so that the sequence encoding the desired antigen is transcribed into RNA in the host tissue transformed by a vector containing this expression construct.

      The choice of control elements will depend on the host being treated and the type of preparation used. Thus, if the host's endogenous transcription and translation machinery will be used to express the proteins, control elements compatible with the particular host will be utilized. In this regard, several promoters for use in  
15        mammalian systems are known in the art and include, but are not limited to, promoters derived from SV40, CMV, HSV, RSV, MMTV, among others.

      In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of antigens encoded by the delivered  
25        nucleotide sequences. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a coding sequence to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other  
30        types of regulatory elements may also be present in the vector, for example, enhancer sequences.

      An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate control and, optionally, regulatory  
35        sequences such that the positioning and orientation of the coding sequence with respect to the control sequences allows the coding sequence to be transcribed under the "control" of the control sequences (i.e., RNA polymerase, which binds to the DNA molecule at the control sequences,  
40        transcribes the coding sequence). Modification of the

5 sequences encoding the particular antigen of interest may  
be desirable to achieve this end. For example, in some  
cases it may be necessary to modify the sequence so that it  
is attached to the control sequences with the appropriate  
orientation; i.e., to maintain the reading frame. The  
10 control sequences and other regulatory sequences may be  
ligated to the coding sequence prior to insertion into a  
vector. Alternatively, the coding sequence can be cloned  
directly into an expression vector which already contains  
the control sequences and an appropriate restriction site.  
15 Conventional mammalian expression vectors and elements  
can be enhanced for use as DNA vaccines. For example, it  
has been found that the addition of signal peptide  
sequences directing secretion of expressed proteins can  
enhance CTL immune response. The use of a Kozak ATG  
20 sequence can enhance the translational efficiency of a DNA  
vaccine. The inclusion of a mono/poly ubiquitination  
sequence in the expression vector can enhance the MHC Class  
I presentation signal while alternatively the use of an  
invariant chain sequence can enhance MHC Class II  
25 presentation signal. The use of such elements is within  
the abilities of those of skill in the art.

#### Administration of Nucleic Acid Preparations

Particle-mediated methods for delivering nucleic acid  
preparations are known in the art. Thus, once prepared and  
30 suitably purified, the above-described nucleic acid  
molecules can be coated onto carrier particles using a  
variety of techniques known in the art. Carrier particles  
are selected from materials which have a suitable density  
in the range of particle sizes typically used for  
35 intracellular delivery from a gene gun device. The optimum  
carrier particle size will, of course, depend on the  
diameter of the target cells.

For the purposes of the invention, tungsten, gold,  
platinum and iridium carrier particles can be used.  
40 Tungsten and gold particles are preferred. Tungsten

5 particles are readily available in average sizes of 0.5 to  
2.0  $\mu\text{m}$  in diameter. Although such particles have optimal  
density for use in particle acceleration delivery methods,  
and allow highly efficient coating with DNA, tungsten may  
10 potentially be toxic to certain cell types and may degrade  
DNA over time. Gold particles or microcrystalline gold  
(e.g., gold powder A1570, available from Engelhard Corp.,  
East Newark, NJ) will also find use with the present  
methods. Gold particles provide uniformity in size  
15 (available from Alpha Chemicals in particle sizes of 1-3  
 $\mu\text{m}$ , or available from Degussa, South Plainfield, NJ in a  
range of particle sizes including 0.95  $\mu\text{m}$ ) and reduced  
toxicity. Microcrystalline gold provides a diverse  
particle size distribution, typically in the range of 0.5-5  
 $\mu\text{m}$ .

20 A number of methods are known and have been described  
for coating or precipitating DNA or RNA onto gold or  
tungsten particles. Most such methods generally combine a  
predetermined amount of gold or tungsten with plasmid DNA,  
CaCl<sub>2</sub>, and spermidine. The resulting solution is vortexed  
25 continually during the coating procedure to ensure  
uniformity of the reaction mixture. After precipitation of  
the nucleic acid, the coated particles can be transferred  
to suitable membranes and allowed to dry prior to use,  
coated onto surfaces of a sample module or cassette, or  
30 loaded into a delivery cassette for use in particular gene  
gun instruments.

#### Administration of Coated Particles

Following their formation, carrier particles coated  
with either nucleic acid preparations, or peptide or  
35 protein antigen preparations, are delivered to mucosal  
tissue using particle-mediated delivery techniques.

Various particle acceleration devices suitable for  
particle-mediated delivery are known in the art, and are  
all suited for use in the practice of the invention.  
40 Current particle acceleration device designs employ an

5 explosive, electric or gaseous discharge to propel coated  
carrier particles toward target cells. The coated carrier  
particles can themselves be releasably attached to a  
movable carrier sheet, or removably attached to a surface  
10 along which a gas stream passes, lifting the particles from  
the surface and accelerating them toward the target. An  
example of a gaseous discharge device is described in U.S.  
Patent No. 5,204,253. An explosive-type device is  
described in U.S. Patent No. 4,945,050. One example of an  
15 electric discharge-type particle acceleration apparatus is  
the ACCELL® instrument (Geniva, Madison, WI), which  
instrument is described in U.S. Patent No. 5,120,657.  
Another electric discharge apparatus suitable for use  
herein is described in U.S. Patent No. 5,149,655. The  
20 disclosure of all of these patents is incorporated herein  
by reference in their entireties.

The coated particles are administered to the subject  
to be treated in a manner compatible with the dosage  
formulation, and in an amount that will be effective to  
bring about a desired immune response. The amount of the  
25 composition to be delivered which, in the case of nucleic  
acid molecules is generally in the range of from 0.001 to  
10.0  $\mu\text{g}$ , more preferably 0.25 to 5.0  $\mu\text{g}$  of nucleic acid  
molecule per dose, depends on the subject to be treated.  
By dose, it is meant to refer to a single event of DNA  
30 delivery by gene gun. Using current gene guns, it is  
common for a single immunization procedure, whether a prime  
immunization or a boost, to include more than one gene gun  
dose. For example, a prime might consist of two to six  
gene gun doses to the tongue. Adding more DNA to each  
35 dose, beyond 0.25 to 5 $\mu\text{g}$ , generally does not increase  
immune response. The additional doses are appropriate to,  
in essence, treat more tissue. A gene gun design which is  
capable of treating more tissue in a single operation would  
lower the number of doses in a single vaccination. In  
40 general, however, the total amount of DNA delivered in the  
entire immunization will be in the range of about 1-30 $\mu\text{g}$

5 total for all doses. Often a prime immunization and either  
one or two boost immunizations will be appropriate to  
achieve the desired level of immune response. The exact  
amount necessary will vary depending on the age and general  
condition of the individual being immunized and the  
10 particular nucleotide sequence or peptide antigens  
selected, as well as other factors. An appropriate  
effective amount can be readily determined by one of skill  
in the art upon reading the instant specification.

In the examples described below, over-dosages of DNA  
15 have been used. This was done because optimization of  
dosages for the particular antigens and the particular  
animals have not yet been done. It has been previously  
found that mild over-dosing of delivered DNA is not harmful  
to the immune response and thus, to err in dosing to  
20 achieve the desired immune response, it was decided to err  
on the high side. For a practical nucleotide vaccine for a  
given antigen, optimization studies would be performed to  
determine the minimum dosing required and such studies are  
well within the skill of those in the art.

25 Thus, an effective amount of the antigens herein  
described, or rather nucleic acids coding therefor, will be  
sufficient to bring about a suitable immune response in an  
immunized subject, and will fall in a single to double  
digit microgram range of DNA that can be optimized through  
30 routine trials for a particular DNA and mammal.

The coated particles are delivered to suitable  
recipient cells in mucosal tissue in order to bring about  
mucosal, humoral and/or cellular immune responses in the  
treated subject.

### 35 C. Experimental

Below are examples of specific embodiments for  
carrying out the present invention. The examples are  
offered for illustrative purposes only, and are not  
intended to limit the scope of the present invention in any  
40 way.

5           Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

10                   Particle-Mediated Nucleic Acid  
                  Immunization Directed to Porcine Mucosal Tissue

In order to assess the effectiveness of particle-mediated nucleic acid immunization of mucosal tissue, the following studies were carried out.

15           Experimental subjects

Weanling pigs (10-15 kg) sero-negative for swine influenza by hemagglutination inhibition (HI), (Palmer et al. (1975) U.S. Department of Health, Education and Welfare Immunology Series) and ELISA (Sheerar (1989) J. Gen. Virol. 70:3297-3303) were housed in a Biosafety level 2-N facility for immunizations, and then housed in a Biosafety level 3-N facility for viral challenge. The animal subjects were cared for in accordance with the guidelines prescribed by the University of Wisconsin Research Animal Resource Center.

Viral preparations

An A/swine influenza isolate, A/Swine/Indiana/1726/88 (H1N1) (Sw/IN), was obtained from the influenza repository at the University of Wisconsin School of Veterinary Medicine. The virus was cultured in 10-day-old embryonated hens' eggs and stored at -70 °C as previously described (Sheerar et al., supra) except that the allantoic fluid was concentrated by the addition of PEG-8000 to 8%. Precipitated virus was centrifuged at 8000 X g prior to purification on 30-60% sucrose gradients at 24,000 rpm in an SW28 rotor (Beckman).

5     Plasmid constructs and DNA preparations

      The hemagglutinin expression plasmid pWRG1638 depicted  
in Figure 1 was constructed by ligating the cloned cDNA  
encoding the HA of swine influenza virus (SW/IN/1726/88)  
into the mammalian expression cassette pWRG7054. The cDNA  
10     synthesis of the HA gene was done in a one-step PCR method  
according to Wentworth et al. (1994) *J. Virol.* 68:2051-  
2058. PWRG1638 is a pUC19-based vector and includes the  
human cytomegalovirus immediate early transcriptional  
enhancer/promoter (CMVie) to drive transcription of the HA  
15     coding region. The plasmid also contains the  
polyadenylation region from the bovine growth hormone bGH  
gene (Chapman et al. (1991) *Nucleic Acids Res.* 19:3979-  
3986). An influenza nucleoprotein (NP) expression plasmid,  
pFluNP, that encodes the nucleoprotein of influenza A  
20     strain PR/8/34 was obtained from Dr. K. Irvine at the  
National Cancer Institute. All plasmids were propagated in  
*E. coli* strain XL1-Blue MR. Supercoiled plasmid DNA was  
prepared on Qiagen columns according to the manufacturer's  
instructions.

25     Preparation of coated microparticles

      Plasmid DNA was coated onto gold particles normally in  
the range of 1-3  $\mu$ m in size (Degussa Corp., South  
Plainfield, NJ) using techniques described by Eisenbraun et  
al. (1993) *DNA Cell Biol.* 12:791-797. The DNA-coated gold  
30     particles were then loaded into Tefzel® tubing as described  
in U.S. Patent No. 5,584,807 to McCabe, and the tubing was  
cut into 1.27 cm lengths to serve as cartridges in the  
ACCELL® gene gun delivery device. The helium-pulse ACCELL®  
gene gun device was obtained from Geniva, Madison, WI. In  
35     the vaccinations, each 1.27 cm cartridge contained 0.5 mg  
gold particles coated with 1.25  $\mu$ g of plasmid DNA.

In vitro expression of HA in CHO cells

      Chinese hamster ovary (CHO) cells were transfected  
with the pWRG1638 construct, or with control plasmid

5 pWRG1630 which codes for the mature form of epidermal  
growth factor (Andree et al. (1994) *Proc. Natl. Acad. Sci.*  
USA 91:12188-12192), using the electric discharge ACCELL®  
gene gun delivery device (Geniva, Madison, WI). In the  
10 study, the CHO cells were cultured as monolayers on 22x22  
mm glass cover slips. For transfection, growth medium was  
aspirated and the cells treated as previously described  
(Christou et al. (1990) *Trends Biotech.* 8:145-151. After  
transfection, fresh medium was added and the cells were  
15 incubated at 37°C overnight. Following incubation, the  
cells were fixed with a methanol/acetone (50:50 v/v) fixing  
solution at -20°C, and then air dried. The fixed cells  
were incubated with a panel of the following monoclonal  
antibodies which are specific for the HA protein of swine  
influenza A (SW/IN/1726/88): 3F2c, 1-6b2, 2-15f1 and 7B1b  
20 (Sheerar et al., *supra*). Incubation was conducted at room  
temperature for 60 minutes, after which the fixed cells  
were washed and incubated with biotinylated goat anti-  
murine antibodies (Oncogene Sciences, Inc.). The cells  
were then washed again, and incubated with fluorescein-  
25 conjugated streptavidin (Oncogene Sciences, Inc.).  
Fluorescently labeled cells were visualized using a  
suitable fluorescence microscope (e.g., a Zeiss  
Photomicroscope III™ equipped for fluorescence microscopy).

As a result of the study, CHO cells that were  
30 transfected with the pWRG1638 construct showed intense  
staining, indicating that the cells were expressing  
influenza HA. CHO cells transfected with the pWRG1630  
control plasmid were not immunoreactive in the assay.

#### In vivo vaccination studies

35 Based on the positive results seen in the above-  
described in vitro transfection study, a vaccination trial  
was initiated using in vivo particle-mediated delivery  
methods. Animal subjects receiving nucleic acid  
immunizations in the present study included: (1) a first  
40 experimental group of three pigs that were vaccinated by



5 particle-mediated delivery to the epidermis with the NP  
expression vector pFluNP; (2) a second experimental group  
of four pigs that were vaccinated by particle-mediated  
10 delivery to the epidermis with the HA expression vector  
pWRG1638; (3) a third experimental group of five pigs that  
were vaccinated by particle-mediated delivery to the  
inferior surface of the tongue (mucosal immunization) with  
the HA expression vector pWRG1638; and (4) a fourth  
15 experimental group of four pigs that were vaccinated by  
particle mediated delivery to the epidermis with a negative  
control plasmid pWRG3510 (a plant expression vector  
encoding  $\beta$ -glucuronidase from *E. coli* and which is inactive  
in mammalian cells). Animals in the first and second  
20 experimental groups were immunized using ACCELL® gene gun  
transfer of either the pFluNP, the pWRG1638 construct, or  
the control plasmid pWRG3510, into the epidermis in  
different anatomical regions including the dorsal surface  
of the ear, the inguinal region, and the lateral thoracic  
25 region. Treatment typically included six target sites at  
each location. Hair was removed with clippers prior to  
treatment of the lateral thoracic region, but other regions  
were treated without prior preparation. Delivery was  
conducted at 500 or 600 psi helium pressure. Animals in  
the third experimental group were immunized using ACCELL®  
30 gene gun transfer of the pWRG1638 construct into the  
mucosal tissue of the inferior surface of the tongue using  
500 or 600 psi driving gas. In other vaccinations, a fifth  
experimental group of four pigs received a 2 ml parenteral  
(intramuscular) injection of a commercial swine influenza  
35 vaccine (MaxiVac™-FLU, SyntroVet, Kenexa, KS) as directed  
by the manufacturer. The MaxiVac™-FLU vaccine is an oil-  
in-water vaccine containing inactivated whole Influenza A  
(H1N1) virus. Vaccination consisted of a priming  
administration followed by a booster injection four weeks  
40 later. A sixth experimental group of four pigs was  
infected with swine influenza and allowed to recover from  
infection to provide a comparison between protection

5       afforded by conventional vaccine and by natural infection.

          In experimental groups 1-5, serum samples were collected prior to vaccination, prior to booster administration, and one week after booster administration. All blood samples were collected from the superior vena  
10       cava. After these serum collections were completed, the animals were challenged with virus, the course of infection monitored, and sera was again collected two weeks after the challenge.

          Viral challenge consisted of intranasal instillation  
15       of  $2 \times 10^4$  or  $2 \times 10^6$  EID<sub>50</sub> (50% egg infectious dose) of SW/IN virus. Challenged animals were monitored daily for clinical signs of influenza infection (e.g., lethargy, coryza and elevated body temperature). Nasal swabs were collected from each pig on days 1, 3, 5, and 7 post  
20       infection, and viral titers were determined by limiting-dilution egg inoculation assays (Wentworth et al. (1994) *J. Virol.* 68:2051-2058). Ten days after challenge, convalescent sera were taken.

          Sera from the various experimental groups were  
25       analyzed by ELISA and HI assays. ELISA serology was conducted using 200 hemagglutinin (HA) units/well of Sarksyl-disrupted purified SW/IN virus diluted in PBS as described (Sheerar et al., *supra*), with the swine antibodies being measured directly using a goat anti-swine  
30       IgG alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD). HI assays were performed using previously described techniques (Palmer et al. (1975), *supra*).

          The results of the ELISA and HI assays for all six  
35       experimental groups are depicted below in Table 1. As can be seen, antibody or HI titers were not detected in any of the experimental groups receiving nucleic acid immunizations four weeks post prime. ELISA titers, ranging from 1:200 to 1:1600, were seen in animals receiving  
40       epidermal vaccinations with the NP (pFluNP) and HA (pWRG1638) expression vectors (experimental groups 1 and 2,

5        respectively) two weeks after the boost, and HI titers  
ranging from 1:10 to 1:160 were seen in the animals of  
group 2 vaccinated with the HA construct (pWRG1638). The  
NP-vaccinated animals (group 1) did not have HI titers,  
despite high ELISA titers, because the HI assay only  
10        detects HA-specific antibodies.

      The animals that received mucosal vaccinations with  
the pWRG1638 HA DNA construct (group 3) had higher ELISA  
titers (ranging from 1:800 to 1:6400), and lower HI titers  
(ranging from 1:20 to 1:80), relative to the animals of  
15        groups 1 and 2 that received epidermal vaccinations. The  
animals of group 5 vaccinated with the inactivated whole  
virus exhibited the highest ELISA and HI titers relative to  
all other experimental groups, while the group receiving  
natural infection (group 6) had ELISA and HI titers similar  
20        to the groups vaccinated with the pWRG1638 HA DNA  
construct. Control animals vaccinated with the plant  
expression vector (group 4) showed no evidence of an anti-  
influenza immune response.

Table 1

Type of Reciprocal Vaccination	Animal Number	4 Week Post-Prime Reciprocal ELISA Titer	4 Week Post-Prime Reciprocal HI Titer	2 Week Post-Boost Reciprocal ELISA Titer	2 Week Post-Boost Reciprocal HI Titer	Post-Challenge HI Titer
Epidermal NP DNA Vaccine	1	<100	<10	1600	<10	20
	2	<100	<10	800	<10	80
	3	<100	<10	1600	<10	80
Epidermal NP DNA Vaccine	1	<100	<10	1600	10	160
	2	<100	<10	800	20	5120
	3	<100	<10	800	160	5120
	4	<100	<10	200	40	1280
Tongue HA DNA Vaccine	1	<100	<10	3200	80	2560
	2	<100	<10	3200	40	5120
	3	<100	<10	1600	20	5120
	4	<100	<10	12800	80	5120
Inactivated Whole Virus	1	6400	160	32000	5120	ND
	2	1600	40	4000	80	ND
	3	6400	80	8000	160	ND
	4	800	40	32000	80	ND
Natural Infection*	1	3200	160	1600	NA	80
	2	800	40	1600	NA	40
	3	1600	160	1600	NA	160
	4	800	20	6400	NA	40
Negative Control	1	<100	<10	<10	<10	40
	2	<100	<10	<10	<10	80
	3	<100	<10	<10	<10	80
	4	<100	<10	<10	<10	80

\*The natural infection cohort was bled three weeks after the first infection and two weeks after the second infection; ND-Not determined; NA-Not applicable

5           As can also be seen by reference to Table 1, the  
animals vaccinated with the pFluNP construct (group 1) and  
the animals treated by "natural infection" had post-  
challenge HI titers ranging from 1:80-1:160. These titers  
are similar to the HI titers seen in the negative control  
10 animals of group 6 that were vaccinated with the pWRG3510  
( $\beta$ -glucuronidase) construct. In contrast, both groups of  
animals that were vaccinated with the pWRG1638 HA DNA  
constructs (groups 2 and 3) had HI titers as high as 1:5120  
after viral challenge. Even the animal from group 2 that  
15 responded poorly to the pre-challenge vaccination in terms  
of HI titer showed some evidence of a hyperimmune response  
following viral challenge.

With respect to the levels of protection afforded by  
the various methods of immunization (e.g., nucleic acid  
20 immunization, parenteral vaccination or viral infection),  
clinical signs of disease (lethargy, coryza and elevated  
body temperature) were monitored during infection, but did  
not provide a reliable measure of disease progression. On  
the other hand, nasal viral titers provided a quantitative  
25 indicator for disease progression.

Referring now to Figure 2, animals vaccinated  
epidermally with the pFluNP DNA construct (group 1)  
developed high antibody titers to NP, but showed no  
evidence of protection from viral infection in terms of  
30 nasal virus titer. Animals receiving epidermal vaccination  
with the pWRG1638 HA DNA construct (group 2) became  
infected and shed lower levels of virus over the course of  
infection, and resolved infection approximately two days  
earlier than the control animals of group 6. Animals  
35 receiving mucosal vaccination with the pWRG1638 HA DNA  
construct (group 3) developed weak HI titers, but were able  
to reduce viral shedding over the seven days of the study.  
Further, the mucosally vaccinated animals were able to  
reduce the initial infection, as evidenced by a decrease in  
40 the level of shedding by an order of magnitude on days 1  
and 3, relative to the epidermally vaccinated animals of

5 group 2.

The animals of group 4 that received the commercial inactivated whole virus vaccine (MaxVac™-FLU) showed the highest titer antibody responses, as seen in the ELISA and HI results of Table 1. However, even though the animals of  
10 group 4 had roughly 1-2 fold higher HI titers relative to the animals receiving nucleic acid immunizations, this higher HI titer did not translate to a higher level of protection upon challenge (Figure 2). In fact, the animal from group 4 having the highest HI titer in the entire  
15 study was the least protected when challenged with the influenza virus.

As a result of the above-described studies, it can be seen that nucleic acid immunization to mucosal tissue via particle-mediated delivery techniques provides an immune  
20 response that is both quantitatively and qualitatively different than the responses generated by particle-mediated epidermal immunization with nucleic acids, or parenteral immunization with inactivated whole virus. Particle-mediated mucosal immunization with the pWRG1638 construct  
25 induced higher ELISA but lower HI influenza-specific antibody titers relative to particle-mediated epidermal immunization with the same construct. Further, the ability of the mucosally vaccinated animals to reduce nasal shedding of virus on days 1 and 3 of infection is  
30 consistent with a systemic mucosal immune response.

### Example 2

#### Particle-Mediated Nucleic Acid

##### Immunization Directed to Equine Mucosal Tissue

The work reported in this example was performed by a  
35 research group separate from that of the inventor here and is reported because it is supportive of the concept of the present invention.

The efficacy of mucosal nucleic acid immunization administered with a gene gun was demonstrated in ponies  
40 using the HA gene of the equine influenza strain

5 A/Equine/Kentucky/A/81, subcloned in a CMV promoter-based eukaryotic expression vector.

Two experimental groups, of four influenza-naive ponies each, were established. The first experimental group received a 3-dose course of particle-mediated nucleic acid immunization to epidermal tissue on days 0, 65 and 130 of the study. The second experimental group received a 3-dose course of particle-mediated nucleic acid immunization to both epidermal and mucosal tissue, also on days 0, 65 and 130 of the study. The mucosal tissue targeted was the lower side of the tongue as well as the conjunctiva and third eyelid of the animals.

Nucleic immunizations were carried out using an ACCELL® (Geniva, Madison, WI) gene gun device.

Each immunization included multiple doses of DNA delivery by gene gun. Each gene gun application delivered .5 µg of DNA. For the epidermal delivery immunizations, each immunization included 14 doses to the inguinal epidermis and 10 doses to the perineum for each animal. For the immunizations to the skin and mucosal tissue, the animals received the same skin doses plus 10 doses to the tongue and 4 doses to the conjunctiva of the third eyelid.

A challenge infection with homologous virus was administered 28 days after the final administration (on day 160 of the study) to each experimental group, and to a third group of four seronegative control ponies.

The results were that all control animals (4/4) showed clinical evidence of influenza virus infection subsequent to challenge, as did 2/4 of the animals from group 1 (receiving epidermal immunizations only). In contrast, none of the animals of group 2 that received both mucosal and epidermal nucleic immunizations (0/4) showed any clinical signs of disease. Results of influenza virus isolation on post-challenge nasal swabs demonstrated that nucleic acid immunization provided complete, or nearly complete protection from infection in two of the four horses that received epidermal and mucosal immunizations.

5

Example 3Particle-Mediated Nucleic Acid Immunization in  
Primates to Immunodeficiency Virus

The efficacy of mucosal nucleic immunization administered with a gene gun was demonstrated in rhesus monkeys. Rhesus monkeys were given a DNA vaccine encoding a tall length gag-pol-envelope construct from simian immunodeficiency virus. The animals were vaccinated on the rectum, the tongue and on the buccal tissue. In each immunization, the gene gun was used to deliver a total of 8  $\mu$ g of DNA to the tongue and cheek (buccal) tissue. Each of the animals received a prime immunization and multiple boost immunizations separated by approximately ninety days or more. In a parallel experiment, monkeys were vaccinated by administering the nucleic acid to the skin using a gene gun. Each animal received multiple boosts to the skin.

Immunized monkeys were tested to determine whether immunization induced a mucosal immune response or a systemic immune responses. The results are presented in Table 2. All four monkeys immunized mucosally showed an increase in CTL response in the mucosal gut tissue. These results indicates that the monkeys vaccinated either in the buccal or tongue tissues were able to elicit a system wide mucosal immune response as demonstrated by the existence of appropriate IgA-based CTL responses in a mucosal site, which was not a site of DNA injection. These results indicate that particle-mediated DNA delivery to mucosal tissue results in more efficient induction of mucosal-specific cellular immune responses than DNA delivery to the skin.

Mucosally-immunized monkeys also demonstrated systemic humoral and cell-mediated immune responses. Two of the three monkeys exhibited increased gag-specific peripheral or humoral blood (PBMC) CTL and three of the four monkeys showed increased IgG titers (200-400). Systemic responses were also observed in monkeys immunized by skin.

The CTL responses in LP were predominantly env-



5 specific. In contrast, peripheral blood CTL responses were  
predominantly gag-specific, regardless of route of DNA  
inoculation. These data suggest that mucosal immunization  
may differ from skin immunization in the predominant  
specificity of the immune responses elicited, because  
10 mucosal immunization was more efficient in eliciting env-  
specific immune responses.

These results indicate that in a variety of mammals  
the delivery of antigen encoding DNA to the mucosal tissues  
of the animal results in an immune response throughout the  
mucosal tissues of the animal even in tissues quite  
15 distance from the site of the DNA delivery. This provides  
a mechanism for generalized development of mucosal immune  
response in mammals through the use of DNA delivery to  
convenient tissues. Obviously, in most mammals, and in  
20 particular people, the most convenient targets for DNA  
delivery would be those which are least invasive, namely  
the tongue and the inside of the cheek.

Accordingly, novel methods for mucosal immunizations  
have been described. Although preferred embodiments of the  
subject invention have been described in some detail, it is  
25 understood that obvious variations can be made without  
departing from the spirit and the scope of the invention as  
defined by the appended claims.

Table 2

Monkey	Immunization site	Gut CTL (env) % specific lysis	PBMC CTL (gag) % specific lysis	Serum IgG (endpoint titer)
L978 (died)	Mucosal	11.5 <sup>a</sup>	dead	400
L999	Mucosal	14.6 <sup>a</sup>	28.0 <sup>d</sup>	200
L775	Mucosal	12.4 <sup>b</sup>	Negative	0
L736	Mucosal	10.0 <sup>b</sup>	18.9 <sup>d</sup>	400
P551	Skin	Negative <sup>**</sup>	38.4 <sup>d</sup>	350 <sup>e</sup>
P194	Skin	Not done	Negative	51200 <sup>e</sup>
M063	Skin	Negative <sup>c</sup>	Negative	102400 <sup>e</sup>
M122	Skin	Negative <sup>c</sup>	7.6 <sup>d</sup>	51200 <sup>e</sup>
P177	Skin	Not done	6.5 <sup>d</sup>	0 <sup>e</sup>
P501	Skin	Not done	18.5 <sup>d</sup>	0 <sup>e</sup>
M223	Skin	18.0 <sup>c</sup>	Negative	0 <sup>e</sup>

\*Negative: % lysis &lt;5.0

<sup>a</sup>Assayed 11/11/96, Post-boost 1 (2 immunizations)<sup>b</sup>Assayed 5/19/97, Post-boost 3 (4 immunizations)<sup>c</sup>Assayed 10/23/97, Post-boost 6 (7 immunizations)<sup>d</sup>Assayed 8/4/98, Post-boost 8 (9 immunizations)<sup>e</sup>Assayed 9/20/96, Post-boost 2, (3 immunizations)

5

## CLAIMS

We claim:

1. A method for inducing a mucosal immune response in a mammal to a pathogen comprising the steps of  
constructing copies of a nucleic acid construct capable of  
10 expressing at least one antigen from the pathogen in the cells of the mammal;  
coating the nucleic acid constructs onto carrier particles;  
accelerating the carrier particles into the cells of  
15 mucosal tissues of the mammal selected from the group consisting of buccal and tongue tissue, the amount of the nucleic acid and the selection of the antigen sufficient to induce a mucosal immune response in the mucosal immune system of the mammal.
- 20 2. A method as claimed in claim 1 wherein the pathogen is a virus.
3. A method as claimed in claim 1 wherein the method includes at least two repetitions at two different times of the step of delivery of the carrier particles into the mammal, one  
25 repetition being a prime immunization and the other being a boost immunization.
4. A method as claimed in claim 1 wherein the nucleic acid is DNA.
5. A method as claimed in claim 1 wherein the total  
30 amount of nucleic acid delivered into the mammal is between about 1 and 30 micrograms.

- 5           6. A method of vaccinating a mammal to impede the  
infection of the mammal by a virus which normally infects  
mammals through mucosal tissue, the method comprising the steps  
of
- 10           making copies of a DNA construct capable of expressing in  
cells of the mammal an antigen from the virus;  
            joining the DNA copies onto carrier particles;  
            physically delivering the carrier particle into the  
interior of cells in the mucosal tissues of the mammal, the  
mucosal tissues of the mammal being selected from the group  
15           consisting of tongue and buccal tissue.
7. A method as claimed in claim 6 wherein the amount of  
DNA delivered into the mucosal cells of the mammal is between  
about 1 and 30 micrograms.
- 20           8. A method as claimed in claim 6 wherein the physical  
delivering step is repeated at least twice at two different  
times, one repeat serving as a prime vaccination and the other  
serving as a boost.
9. A method for inducing both a mucosal immune response  
and a cytotoxic immune response in a mammal to a pathogen  
25           comprising the steps of:  
            constructing copies of a nucleic acid construct capable of  
expressing at least one antigen from the pathogen in the cells  
of the mammal;  
            coating the nucleic acid constructs onto carrier  
30           particles;  
            accelerating the carrier particles into the cells of  
mucosal tissue of the mammal, the mucosal tissue selected from  
the group consisting of buccal and tongue tissue, the amount of  
the nucleic acid and the selection of the antigen sufficient to  
35           induce an immune response in the mammal.

5           10. A method as claimed in claim 9, wherein the step of accelerating the carrier particles into the cells of the mammal is repeated one or more times.

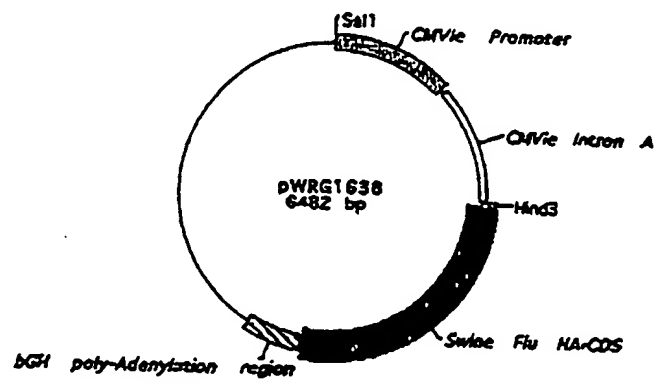
          11. A method as claimed in claim 9 wherein the pathogen is a virus.

10           12. A method as claimed in claim 9 wherein the method includes at least two repetitions at two different times of the step of delivery of the carrier particles into the mammal, one repetition being a prime immunization and the other being a boost immunization.

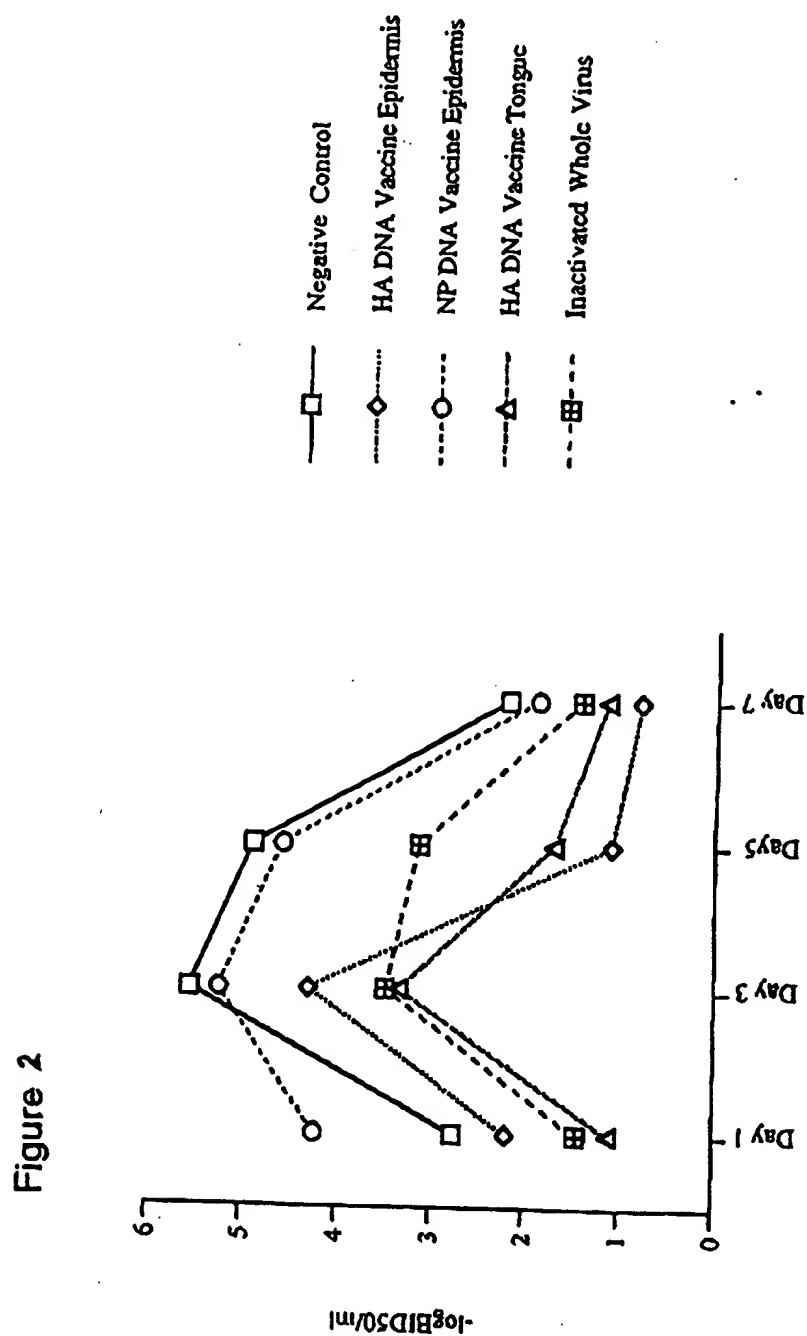
15           13. A method as claimed in claim 9 wherein the nucleic acid is DNA.

          14. A method as claimed in claim 9, wherein the amount of nucleic acid delivered into the mammal is between about 1 and 30 micrograms.

Figure 1



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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/17637

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70; C12N 15/00, 15/63; C12P 21/00

US CL :435/69.1, 172.1, 172.3; 514/44; 935/52

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.1, 172.3; 514/44; 935/52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STAATS et al. Mucosal immunity to infection with implications for vaccine development. Curr. Opin. Immunol. August 1994, Vol. 6, No. 4, pages 572-583, see entire document.	1-14
Y	KELLER et al. In vivo particle-mediated cytokine gene transfer into canine oral mucosa and epidermis. Cancer Gene Therapy. June 1996, Vol. 3, No. 3, pages 186-191, see entire document.	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 OCTOBER 1998

Date of mailing of the international search report

26 OCT 1998

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/17637

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN:Medline, Biosis, CAPlus, Embase, WPIDS

APS

Search Terms: mucosal immunization, DNA vaccine, genetic vaccine, vector, antigen, muoosa

